Calmodulin-dependent protein phosphatase has been proposed to be an important phosphotyrosyl-protein phosphatase. The ability of the enzyme to attack autophosphorylated insulin receptor was examined and compared with the known ability of the enzyme to act on autophosphorylated epidermal-growth-factor (EGF) receptor. Purified calmodulin-dependent protein phosphatase was shown to catalyse the complete dephosphorylation of phosphotyrosyl-(insulin receptor). When compared at similar concentrations, $^{32}\text{P}$-labelled EGF receptor was dephosphorylated at $>3$ times the rate of $^{32}\text{P}$-labelled insulin receptor; both dephosphorylations exhibited similar dependence on metal ions and calmodulin. Native phosphotyrosyl-protein phosphatases in cell extracts were also characterized. With rat liver, heart or brain, most (75%) of the native phosphatase activity against both $^{32}\text{P}$-labelled insulin and EGF receptors was recovered in the particulate fraction of the cell, with only 25% in the soluble fraction. This subcellular distribution contrasts with results of previous studies using artificial substrates, which found most of the phosphotyrosyl-protein phosphatase activity in the soluble fraction of the cell. Properties of particulate and soluble phosphatase activity against $^{32}\text{P}$-labelled insulin and EGF receptors are reported. The contribution of calmodulin-dependent protein phosphatase activity to phosphotyrosyl-protein phosphatase activity in cell fractions was determined by utilizing the unique metal-ion dependence of calmodulin-dependent protein phosphatase. Whereas Ni$^{2+}$ (1 mM) markedly activated the calmodulin-dependent protein phosphatase, it was found to inhibit potently both particulate and soluble phosphotyrosyl-protein phosphatase activity. In fractions from rat liver, brain and heart, total phosphotyrosyl-protein phosphatase activity against both $^{32}\text{P}$-labelled receptors was inhibited by 99.5 ± 6% (mean ± s.e.m., 30 observations) by Ni$^{2+}$. Results of Ni$^{2+}$ inhibition studies were confirmed by other methods. It is concluded that in cell extracts phosphotyrosyl-protein phosphatases other than calmodulin-dependent protein phosphatase are the major phosphotyrosyl-(insulin receptor) and -(EGF receptor) phosphatases.

INTRODUCTION

Insulin is believed to elicit many of its actions by stimulating a tyrosine protein kinase activity intrinsic to the $\beta$-subunit of the insulin receptor (Kasuga et al., 1982; Ulrich et al., 1985; Ebina et al., 1987; Morgan & Roth, 1987; Chou et al., 1987; for review see Sale, 1988). This leads to autophosphorylation of the $\beta$-subunit on tyrosine and phosphorylation of other proteins (White et al., 1985; Haring et al., 1987). Autophosphorylation increases the receptor tyrosine kinase activity towards exogenous substrates. This activated state of the receptor is maintained even after removal of bound insulin (Rosen et al., 1983; Yu & Czech, 1984). Thus dephosphorylation, and not simply dissociation of bound insulin, is required to terminate tyrosine kinase activity. Consequently the phosphotyrosyl-protein phosphatase(s) which catalyses dephosphorylation of the insulin receptor and cellular substrates phosphorylated on tyrosine plays a crucial role in terminating the insulin signal. Such phosphatases could also be subject to regulation and could thus play a key functional role in the overall scheme of insulin action.

At present, none of the native phosphotyrosyl-protein phosphatases active against either the insulin receptor or other tyrosine kinase-containing receptors and oncogene products have been purified to homogeneity and characterized; nor indeed is it known how many species of phosphatase account for all the phosphotyrosyl dephosphorylations of these various substrates. There have been no reports in which the insulin receptor has been used as substrate to characterize phosphotyrosyl-protein phosphatase(s). Previous studies in other systems have resulted in partial purifications of certain phosphotyrosyl-protein phosphatases, mainly by using artificial substrates such as phosphotyrosyl-(bovine serum albumin) (Foulkes et al., 1981, 1983; Chernoff & Li, 1983; Shriner & Brautigan, 1984; Okada et al., 1986; Rotenberg & Brautigan, 1987). These substrates are far from ideal; e.g. phosphotyrosine may not be present within protein sequences representative of those found in the native receptors or oncogene products. Thus phosphatases with the greatest activity and specificity toward the receptor substrate or oncogene product of interest may be missed. Consequently, in the present work we have employed native autophosphorylated insulin receptor as substrate. The system used also contains the EGF receptor and thus affords the opportunity for the simultaneous study of phosphotyrosyl-protein phosphatases active against both autophosphorylated insulin and EGF receptors. The system has been used in the present paper in two ways: firstly, to study the role of a known phosphatase, calmodulin-dependent protein phosphatase, and secondly to compare the properties and subcellular distributions of native cellular phosphatases active against the two receptors.

Abbreviation used: EGF, epidermal growth factor.
Calmodulin-dependent protein phosphatase, also called protein phosphatase 2B or calcineurin, has emerged as a candidate for being a cellular phosphotyrosyl-protein phosphatase. The enzyme has been observed to dephosphorylate phosphotyrosyl-(EGF receptor) (Pallen et al., 1985) and under certain conditions phosphotyrosyl residues with equal efficacy to phosphoserine residues in synthetic peptides containing either a phosphotyrosyl or phosphoserine residue present in an identical peptide sequence (Chan et al., 1986). Thus it is important to test whether the phosphatase acts on other phosphotyrosyl proteins and to analyse its physiological role as a phosphotyrosyl-protein phosphatase. In the present work we have investigated whether autophosphorylated insulin receptor is a substrate for calmodulin-dependent protein phosphatase, and assessed the contribution of the phosphatase to total phosphotyrosyl-protein phosphatase activity in cell extracts against auto-phosphorylated insulin and EGF receptors.

EXPERIMENTAL

Materials

Leupeptin, pepstatin A, antipain, α-casein and trifluoperazine were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Calmodulin-dependent protein phosphatase purified from bovine brain and calmodulin were generously given by Dr. C. R. Klee (National Cancer Institute, Bethesda, MD, U.S.A.). Sephadex G-50 came from Pharmacia Fine Chemicals, Uppsala, Sweden. Soya-bean trypsin inhibitor was from BDH Chemicals, Poole, Dorset, U.K. Other chemicals and biochemicals were obtained from sources described in Smith et al. (1988).

Preparation of phosphorylated insulin and EGF receptors

Insulin and EGF receptors were co-purified from solubilized human placental membranes by affinity chromatography on wheat-germ-agglutinin–agarose as previously described (Pike et al., 1984; Smith et al., 1988). The receptors were preincubated (approx. 1 mg of protein/ml) for 15 min at 22°C in 0.15–0.45 ml of 50 mM-Hepes (pH 7.4)/0.1% Triton X-100 in the presence of 2 mM-MnCl₂, 10 mM-MgCl₂ and 100 mM-insulin (Sale et al., 1986). Phosphorylation reactions were initiated by addition of [γ-³²P]ATP (100 μM, 5–30 c.p.m./fmol) and were allowed to proceed at 22°C for 10 min. Excess [γ-³²P]ATP and metal ions that would interfere with phosphatase assays were then removed by rapid gel filtration at 4°C on a small Sephadex G-50 column (2.5–4.5 ml) equilibrated in 50 mM-Hepes (pH 7.4)/0.1% Triton X-100.

Dephosphorylation assays

³²P-labelled receptors (0.8 mg of protein/ml) were incubated at 30°C without or with phosphatase (5 μl of particulate or soluble fractions, or 80 μg of purified calmodulin-dependent protein phosphatase/ml). Incubations were carried out in 50 mM-Hepes (pH 7.4)/0.09% Triton X-100, with additions of free metal ions to the concentrations stated, and calmodulin and EDTA as indicated, in a final volume of 30–40 μl. In assays in which the activity of calmodulin-dependent phosphatase was measured, phosphatase was first preincubated for up to 20 min at 30°C with the same additions of metal ions, calmodulin or EDTA as present in the assay. Dephosphorylations were terminated at the times indicated by adding 0.25 vol. of 5-fold-concentrated Laemmli (1970) sample buffer (100 mM-Tris/HCl, pH 7.4, 10% SDS, 75 mg of dithiothreitol/ml, 0.1% Bromophenol Blue) and heating the samples at 100°C for 2 min. Sucrose was added to a final concentration of 200 mg/ml and samples were analysed by SDS/polyacrylamide-gel electrophoresis on 4%-acylamide stacking and 7.5%-acylamide resolving gels (Laemmli, 1970). Electrophoresis was performed at 20°C for approx. 3 h at 30–35 mA. Gels were stained for 20 min with 0.25% Coomassie Brilliant Blue in 50% (w/v) trichloroacetic acid, destained overnight in 5% acetic acid/45% methanol (both v/v) and dried in vacuo for 1 h at 80°C. Autoradiography, measurement of ³²P associated with appropriate bands and phosphoamino acid analysis were performed as described previously (Smith et al., 1988).

Assay of purified calmodulin-dependent protein phosphatase with p-nitrophenol phosphate as substrate was performed as described previously (Pallen & Wang, 1983; Pallen et al., 1985). The phosphatase activated by Mn³⁺/Ca²⁺/calmodulin in the present work had a specific activity against p-nitrophenol phosphate of 1174 nmol/min per mg in the presence of 0.09% Triton X-100. This specific activity is comparable with previously reported values for the purified phosphatase with this substrate (Pallen & Wang, 1983; Pallen et al., 1985).

Preparation of particulate and soluble fractions

All operations were performed at 4°C. The liver (8–12 g) from one male Wistar rat (200–300 g) was homogenized for 2×15 s in a motor-driven Teflon/glass homogenizer in 50 mM-Hepes (pH 7.0; 2 ml/g), containing 5 mM-EDTA, 0.5 mM-EGTA, 1 mM-dithiothreitol, a 1:1000 dilution of 2.5% (v/v) phenylmethyl-sulphonyl fluoride dissolved in propan-2-ol, and 1 μg each of leupeptin, antipain, pepstatin A and soya-bean trypsin inhibitor/ml. The homogenate was centrifuged at 500 g for 5 min. The particulate and soluble fractions were isolated by centrifuging the 500 g supernatant at 100000 g for 1 h. The supernatant obtained represents the soluble fraction. The particulate fraction was obtained by washing and resuspending the pellet in the above buffer. Both soluble and particulate fractions were usually adjusted to give a protein concentration of 5.5 mg/ml (biuret), and 5 μl was used in the dephosphorylation assays except where stated otherwise. In experiments examining the effect of EDTA on phosphatase activity EDTA and EGTA were omitted; this did not affect recovery of phosphatase activity. Preparation of particulate and soluble fractions from other rat tissues followed a similar protocol.

RESULTS

Phosphotyrosyl-protein phosphatases active against the insulin and EGF receptor were compared by using wheat-germ-lectin-Sepharose-purified preparations of the solubilized receptors from human placenta membranes. Preparations were first phosphorylated by incubation with Mg³⁺, Mn³⁺ and [γ-³²P]ATP in the presence of insulin. Excess [γ-³²P]ATP and metal ions that would interfere with phosphatase assays were then removed by rapid gel filtration on a short G-50 column. Analysis of
the resulting preparations by SDS/polyacrylamide-gel electrophoresis showed the presence of two major \(^{32}\text{P}\)-labelled bands, of \(M_r\) 95000 (autophosphorylated insulin receptor \(\beta\)-subunit) and \(M_r\) 170000 (autophosphorylated EGF receptor) (Pike et al., 1984; Smith et al., 1988). Identity of the \(^{32}\text{P}\)-labelled bands was confirmed by showing insulin or EGF stimulation of phosphorylation of the \(M_r\)-95000 and \(M_r\)-170000 bands respectively, and by using specific antibodies (results not shown). The phosphorylation of the receptors was routinely performed in the presence of insulin but in the absence of EGF, because this gave an approximately equal labelling intensity of the two receptors. EGF is known only to alter the rate of phosphorylation of the same phosphorylation sites in the EGF receptor (Downward et al., 1984). Excision of the \(M_r\)-170000 or \(M_r\)-95000 bands and phosphoamino acid analysis showed that \(> 95\%\) and approx. \(80\%\) respectively, of \(^{32}\text{P}\) recovered in phosphoamino acids was attached to tyrosine (e.g. see Fig. 3). The preparation was essentially devoid of phosphotyrosyl-protein phosphatase activity (0–1 % dephosphorylation/ min). Thus two suitable substrates for assaying phosphotyrosyl-protein phosphatase activity have been copurified. This system has been used in two ways to study phosphotyrosyl-protein phosphatases: firstly, to study a known phosphatase, calmodulin-dependent protein phosphatase, and secondly to study phosphatases in cell extracts. In both cases, \(^{32}\text{P}\) associated with the receptors was determined after separation of proteins by SDS/polyacrylamide-gel electrophoresis. Provided that the extent of receptor dephosphorylation did not exceed \(40\%\) under the assay conditions used, the extent of dephosphorylation was acceptably proportional to the amount of phosphatase activity added (e.g. see Fig. 4b for exact relationship). As the extent of dephosphorylation increased above \(40\%\), disproportionality became increasingly evident. Thus in assays comparing the amount of phosphatase activity, the amount of phosphatase activity added was routinely restricted to that required to elicit \(< 40\%\) dephosphorylation.

**Calmodulin-dependent protein phosphatase**

Calmodulin-dependent protein phosphatase was routinely activated by preincubation with Ca\(^{2+}\) (1 mM)/Mn\(^{2+}\) (1 mM)/calmodulin (1 \(\mu\)m) and then incubated with \(^{32}\text{P}\)-labelled receptors under similar conditions. The activated phosphatase was shown for the first time to be capable of completely dephosphorylating phosphotyrosyl-insulin receptor (Fig. 1). Phosphotyrosyl-(EGF receptor) was also shown to be dephosphorylated by calmodulin-dependent protein phosphatase, confirming observations by Pallen et al. (1985). The dependence of calmodulin-dependent protein phosphatase activity against \(^{32}\text{P}\)-labelled receptors on metal ions and calmodulin was characterized (Table 1). The phosphatase was inactive in the presence of 1 mM-EDTA and required metal ions for activity. Phosphatase activity against both \(^{32}\text{P}\)-labelled insulin and EGF receptors changed in parallel with a variety of activators. The enzyme was directly activated by Ni\(^{2+}\) and Mn\(^{2+}\) (Ni\(^{2+}\) > Mn\(^{2+}\)) and further stimulated by calmodulin, whereas the enzyme was only activated by Ca\(^{2+}\) in the presence of calmodulin. This profile of metal-ion and calmodulin dependence is exactly that expected of calmodulin-dependent protein phosphatase (Stewart et al., 1982, 1983; Pallen & Wang, Vol. 256

![Fig. 1. Dephosphorylation of \(^{32}\text{P}\)-labelled insulin and EGF receptors by calmodulin-dependent protein phosphatase; effects of vanadate and Zn\(^{2+}\)](image)

Calmodulin-dependent protein phosphatase was activated by preincubation at 30 °C for 20 min with 1 mM-Mn\(^{2+}\)/1 mM-Ca\(^{2+}\)/1 \(\mu\)m-calmodulin. \(^{32}\text{P}\)-labelled receptors were incubated for 1 h at 30 °C with 1 mM-Mn\(^{2+}\)/1 mM-calmodulin with or without activated calmodulin-dependent protein phosphatase, Zn\(^{2+}\) (500 \(\mu\)m) and vanadate (20 \(\mu\)m) as indicated. The samples were then subjected to SDS/polyacrylamide-gel electrophoresis. An autoradiograph of the result is shown.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Insulin receptor</th>
<th>EGF receptor</th>
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<tr>
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<td>100</td>
</tr>
<tr>
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<tr>
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</tr>
<tr>
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phosphotyrosyl-protein phosphatase inhibitors

insulin and EGF
calmodulin-dependent protein

then inhibited the dephosphorylation of both \(^{32}\)P-labelled insulin and EGF receptors catalysed by Mn\(^{2+}/Ca^{2+}/calmodulin-activated phosphatase (Fig. 1). Fig. 2 shows the time course of dephosphorylation of \(^{32}\)P-labelled insulin and EGF receptors. When similar amounts of \(^{32}\)P were present in EGF and insulin receptors, the \(^{32}\)P-labelled EGF receptor was dephosphorylated at \(>3\) times the rate of the \(^{32}\)P-labelled insulin receptor. Insulin receptor isolated by the protocol employed was phosphorylated on both tyrosine and serine residues in the presence of insulin (Fig. 3; Smith et al., 1988), whereas the EGF receptor was \(>95\%\) phosphorylated on tyrosine. Phosphoamino acid analysis confirmed that calmodulin-dependent protein phosphatase dephosphorylated phosphotyrosyl residues in the phospho-(insulin receptors) (Fig. 3). Thus these results establish in vitro that the calmodulin-dependent protein phosphatase dephosphorylates insulin receptor auto-phosphorylated on tyrosine residues. Calmodulin-dependent protein phosphatase also dephosphorylated phosphoseryl residues of the phospho-(insulin receptor).

**Cellular phosphotyrosyl-protein phosphatases**

By using \(^{32}\)P-labelled insulin and EGF receptors as substrate, phosphotyrosyl-protein phosphatase activity was detected in extracts of a variety of tissues. In the rat it occurred in the order (per g of tissue) kidney > liver > heart > brain > skeletal muscle > adipose tissue with either substrate (ratio 1:0.83:0.45:0.20:0.08:0.05). Phosphotyrosyl-protein phosphatase activity was completely inhibited by Zn\(^{2+}(500 \mu M)\) or vanadate (20 \(\mu M\)), showing that loss of \(^{32}\)P from the receptors was genuinely due to phosphatase activity and not to proteolytic degradation of the receptors. Additionally, \(M_s\) values of \(^{32}\)P-labelled insulin and EGF receptor, determined by SDS/polyacrylamide-gel electrophoresis, were unaltered during incubation with extracts. As liver is a well-characterized target of insulin action and contained almost as high a phosphotyrosyl-protein phosphatase activity as kidney, it was used as the main source of phosphotyrosyl-protein phosphatase activity in further studies.

Subcellular-fractionation studies were conducted initially to determine the distribution of phosphotyrosyl-protein phosphatase activity between particulate and soluble fractions of the cells. A detailed analysis using

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*Fig. 2. Time courses of dephosphorylation of \(^{32}\)P-labelled insulin and EGF receptor by calmodulin-dependent protein phosphatase*

Calmodulin-dependent protein phosphatase was activated and incubated with \(^{32}\)P-labelled receptors for various times as in the legend to Fig. 1. Samples were subjected to SDS/polyacrylamide-gel electrophoresis and autoradiography (a). The extent of phosphorylation of the insulin receptor (●) and the EGF receptor (○) was measured by counting excised bands for radioactivity (b).

*Fig. 3. Phosphoamino acid analysis of \(^{32}\)P-labelled insulin and EGF receptors incubated with and without calmodulin-dependent protein phosphatase*

Calmodulin-dependent protein phosphatase was activated and incubated with \(^{32}\)P-labelled receptors for 20 min as in the legend to Fig. 1. After SDS/polyacrylamide-gel electrophoresis the \(\beta\)-subunit of the insulin receptor (a) and the EGF receptor (b) were subjected to phosphoamino acid analysis and autoradiography.
various amounts of liver particulate and soluble fractions is shown in Fig. 4. About 3 times as much soluble extract was required to produce the same degree of dephosphorylation of either $^{32}$P-labelled insulin or EGF receptor compared with the particulate extract. For example, 30 $\mu$g of soluble and 10 $\mu$g of particulate extract were required to produce 40% dephosphorylation of $^{32}$P-labelled insulin receptor in 10 min. Total yields of soluble and particulate fractions from one rat liver in the experiment of Fig. 4 were 338 mg and 353 mg respectively. Thus 75% of phosphotyrosyl-protein phosphatase activity towards either $^{32}$P-labelled insulin or EGF receptors was membrane-associated, and 25% was recovered in the cytosol. This distribution was confirmed in experiments on a further seven preparations from rat liver and also for preparations from rat heart and brain.

When approximately equal amounts of $^{32}$P were present in EGF and insulin receptors, the $^{32}$P-labelled EGF receptor was dephosphorylated at twice the rate of the $^{32}$P-labelled insulin receptor by both liver particulate and soluble fractions (Fig. 5). Similar results were obtained for rat kidney, heart, brain, skeletal muscle and adipose tissue. Phosphoamino acid analysis confirmed rapid dephosphorylation with time of $^{32}$P-phosphotyrosine from both receptors catalysed by the liver particulate and soluble fractions. The $^{32}$P-labelled EGF receptor used was essentially devoid of $^{32}$P-phosphoserine. However, the $^{32}$P-labelled insulin receptor contained some $^{32}$P-phosphoserine. Under the assay conditions used, dephosphorylation of $^{32}$P-phosphoserine present in the insulin receptor was > 15% over the 10 min incubation period (Fig. 5).

Effects of various agents on liver cytosolic and particulate phosphatase activity against $^{32}$P-labelled insulin and EGF receptors were examined. In each case phosphotyrosyl-protein phosphatase activities were similar in the presence or absence of 1 mM-EDTA (results not shown), but were slightly inhibited by 1 mM-Mn$^{2+}$ (20 ± 2%; mean ± S.E.M., 8 observations); 1 mM-Mg$^{2+}$ and 1 mM-Ca$^{2+}$ had no consistent effects (Fig. 6).

**Fig. 4. Determination of the subcellular distribution of phosphotyrosyl-protein phosphatase activity with $^{32}$P-labelled insulin and EGF receptors as substrates**

Particulate (353 mg of protein) and soluble (338 mg of protein) fractions were prepared from one rat liver. Various amounts of each fraction (0–117 $\mu$g) were incubated with $^{32}$P-labelled receptors in a final volume of 35 $\mu$l for 10 min at 30°C. Samples were subjected to SDS/polyacrylamide-gel electrophoresis and autoradiography (a). The extent of dephosphorylation of receptors was determined by measurement of radioactivity in excised bands (b): Ⓐ, Ⓑ, EGF receptor; Ⓒ, Ⓓ, insulin receptor; Ⓐ, Ⓓ, particulate fraction; Ⓕ, Ⓕ, soluble fraction.

The amount of phosphotyrosyl-protein phosphatase activity in the particulate and soluble fractions attribu-
M. J. King and G. J. Sale

(a) Particulate fraction

Time (min) 0 5 10 15 20
Soluble fraction 20 15 10 5 0

Fig. 5. Time courses of dephosphorylation of $^{32}$P-labelled insulin and EGF receptor by particulate and soluble fractions from rat liver

$^{32}$P-labelled insulin and EGF receptors were incubated for various times with 27.5 $\mu$g of particulate or soluble fraction in a final volume of 35 $\mu$l. (a) Samples were analysed by SDS/polyacrylamide-gel electrophoresis and autoradiography. (b) EGF receptor and insulin receptor $\beta$-subunits were excised from the gel, counted for radioactivity (○) and subjected to phosphoamino acid analysis. Radioactivity recovered in phosphotyrosine (●) and phosphoserine (□) was determined.

table to calmodulin-dependent protein phosphatase was assessed by making use of the unique metal-ion dependence of calmodulin-dependent protein phosphatase. It was observed that, whereas Ni$^{2+}$ (1 mM) markedly activated calmodulin-dependent protein phosphatase to twice the physiological maximum achievable with Ca$^{2+}$ and calmodulin, Ni$^{2+}$ (1 mM) inhibited phosphotyrosyl-protein phosphatase activity in cell fractions (Table 1, Fig. 6). The amount of phosphotyrosyl-protein phosphatase activity not inhibited by Ni$^{2+}$ represents an upper limit for the amount due to calmodulin-dependent protein phosphatase. In particulate and soluble fractions from liver, phosphatase activity towards both $^{32}$P-labelled insulin and EGF receptors was inhibited 100% by 1 mM-Ni$^{2+}$ (102 ± 6%; mean ± S.E.M., 22 observations); e.g. see Fig. 6. Similarly, total phospho-

(b) Particulate fraction

EGF receptor

$\beta$-subunit of insulin receptor

Soluble fraction

Time (min) 0 5 10 15 20
Insulin receptor 20 15 10 5 0

Fig. 6. Comparison of effects of Ni$^{2+}$ and Mn$^{2+}$/Ca$^{2+}$/calmodulin on calmodulin-dependent protein phosphatase activity and phosphotyrosyl-protein phosphatase activity in liver extracts

$^{32}$P-labelled receptors were incubated in a final volume of 35 $\mu$l for 10 min at 30 °C with either no addition (control, CON) or the indicated additions of calmodulin-dependent protein phosphatase (80 $\mu$g/ml), particulate extract (27.5 $\mu$g), soluble extract (27.5 $\mu$g), metal ions (1 mM), EDTA (1 mM), calmodulin (CaM; 1 $\mu$M) or sodium vanadate (20 $\mu$M). Calmodulin-dependent protein phosphatase and cell extracts were first preincubated for 10 min at 30 °C with the same mixture of metal ions, EDTA and calmodulin as used in the incubations. Samples were subject to SDS/polyacrylamide-gel electrophoresis, followed by densitometric scanning of autoradiographs. (a) Calmodulin-dependent protein phosphatase; results are from two separate experiments. (b) Particulate extract. (c) Soluble extract.
tyrosyl-protein phosphatase activity against 32P-labelled insulin and EGF receptors determined in subcellular fractions from brain (which has been reported to contain the highest concentration of calmodulin-dependent protein phosphatase activity; Wallace et al., 1980) and heart was inhibited by 90–100% by 1 mM-Ni2+ (results not shown). Therefore, on the basis of studies with Ni2+, calmodulin-dependent protein phosphatase does not contribute significantly to phosphotyrosyl-protein phosphatase activity in extracts from rat liver, heart and brain.

In a second approach towards determining the contribution of calmodulin-dependent protein phosphatase to phosphotyrosyl-protein phosphatase activity, use was made of the facts that calmodulin-dependent protein phosphatase is inactive in the presence of 1 mM-EDTA, and is highly active in the presence of 1 mM-Mn2+/1 mM-Ca2+/1 μM-calmodulin (Table 1, Fig. 6). Therefore an estimate of the relative amount of phosphotyrosyl-protein phosphatase due to calmodulin-dependent protein phosphatase can be obtained by comparing the activity in the presence of EDTA with that in the presence of Mn2+/Ca2+/calmodulin. Activity of phosphotyrosyl-protein phosphatase in liver particulate and soluble fractions against 32P-labelled insulin or EGF receptors was high in the presence of EDTA and was not further increased by Mn2+/Ca2+/calmodulin (Fig. 6). In fact, Mn2+/Ca2+/calmodulin caused a slight inhibition (25%), owing to the inhibitory action of Mn2+, and gave phosphatase activities identical with that measured with Mn2+ alone. Because calmodulin-dependent protein phosphatase is only partially active with Mn2+, and is stimulated a further 3–4-fold on addition of Ca2+/calmodulin, the lack of any difference between effects of Mn2+ and Mn2+/Ca2+/calmodulin showed that the contribution of calmodulin-dependent protein phosphatase to phosphotyrosyl-(insulin receptor) and -(EGF receptor) phosphatase activity was low. Similar results were obtained in extracts from heart and brain. Thus results with Mn2+/Ca2+/calmodulin support those obtained with Ni2+.

Both results obtained with Ni2+ and Mn2+/Ca2+/calmodulin were validated by performing de-phosphorylation assays over a wider range of 32P-labelled insulin and EGF receptor dephosphorylation than those shown in Fig. 6 (i.e. removal of 20–90% of the 32P; results not shown). Although calmodulin-dependent protein phosphatase is well known to be stable in the buffer used to prepare extracts (i.e. in the presence of EDTA/EGTA/proteinase inhibitors; see, e.g., Klee et al., 1983), there has been a report that the enzyme in skeletal-muscle extracts is unstable on incubation with Mn2+ or Ca2+ in the absence of proteinase inhibitors (Stewart et al., 1983). In the present work proteinase inhibitors were present throughout preincubation and incubation of extracts. To exclude further the possibility that proteolysis of calmodulin-dependent phosphatase during preincubation or incubation of extracts was interfering with the results, two types of experiment were conducted. Firstly, careful analysis by the procedure of Stewart et al. (1983) was undertaken. This procedure is designed for assaying calmodulin-dependent protein phosphatase in cell extracts and minimizing proteolysis. Thus, shorter preincubation/incubation times than those used in Fig. 6, including omitting preincubation altogether, were tried. Under all conditions Ni2+ fully inhibited and Mn2+/Ca2+/calmodulin failed to increase phosphotyrosyl-protein phosphatase activities. Secondly, to provide a positive control, calmodulin-dependent protein phosphatase was assayed in extracts with [32P]phosphoserine as substrate (0.2 mg/ml, 1–2 min incubations; Tallant & Cheung, 1984; Chernoff et al., 1984). Assay of calmodulin-dependent protein phosphatase in extracts is complicated by a high background of activity due to other phosphoserine-protein phosphatases. This was overcome by selectively assaying calmodulin-dependent protein phosphatase, based on its inhibition by trifluoperazine (Stewart et al., 1983). Extracts from brain, liver and heart were incubated with 1 mM-Ca2+ and 1 μM-calmodulin, with or without 100 μM-trifluoperazine. Trifluoperazine-sensitive activity was approx. 35% (brain), 12% (liver) and 10% (heart) of total activity, and was > 80% stable during incubation at 30 °C for 20 min. The proportion of activity inhibited by trifluoperazine in brain extracts is similar to that reported by Tallant et al. (1983), although values for all three tissues are somewhat higher than those evident from the data of Ingebritsen et al. (1983), presumably reflecting the use of a different substrate.

In previous studies, high activities of phosphotyrosyl-casein, -albumin or -IgG phosphatase were noted in the presence of EDTA or EDTA/NaF. These studies did not, however, exclude a contribution of calmodulin-dependent protein phosphatase to the phosphotyrosyl-protein phosphatase activity, because comparative measurements were not made under conditions whereby calmodulin-dependent protein phosphatase was highly active (Brautigan et al., 1981; Foulkes et al., 1983; Chernoff & Li, 1983; Shriner & Brautigan, 1984). Although Foulkes et al. (1983) compared phosphotyrosylcasein phosphatase activities in brain extracts obtained in the presence of EDTA with those in the presence of Mn2+, Mn2+ inhibited the phosphatase activity by 50%, which would have obscured any contribution by partially activated calmodulin-dependent protein phosphatase.

DISCUSSION

An understanding of the properties and regulation of insulin-receptor phosphotyrosyl-protein phosphatases is necessary for a complete description of the mechanism of insulin action. In the present work phosphotyrosyl-protein phosphatases active against autophosphorylated insulin receptors were studied and compared with the properties of those active against autophosphorylated EGF receptors. Purified calmodulin-dependent protein phosphatase catalysed dephosphorylation of both receptors. No major differences in the profile of calmodulin-dependent protein phosphatase activity with various metal ions and calmodulin was found for the two substrates.

To clarify the role of calmodulin-dependent protein phosphatase further, native phosphotyrosyl-protein phosphatase(s) active against autophosphorylated insulin and EGF receptor were first characterized. With rat liver, heart or brain, 75% of phosphotyrosyl-protein phosphatase activity was found in the particulate fraction of the cell and 25% in the soluble fraction with either receptor substrate. This subcellular distribution contrasts with results of previous studies, which have principally resulted in a partial characterization of cytosolic phosphatases (Foulkes et al., 1981, 1983; Chernoff & Li,
Insulin-receptor phosphotyrosyl-protein phosphatases

1983; Shriner & Brautigan, 1984; Okada et al., 1986) and found no more than 36–45% of the phosphatase in membrane fractions (Martensen, 1982; Nelson & Branton, 1984; Rotenberg & Brautigan, 1987). This difference may be attributable to the use of non-specific artificial substrates in the previous studies, whereas specific receptor substrates have been utilized in the present work. Determination of the precise subcellular location of the particulate enzyme is clearly important, and requires further study. Particulate and soluble phosphotyrosyl-protein phosphatase activities against either receptor were inhibited by vanadate and were active in the presence of EDTA, distinguishing them from acid and alkaline phosphatase (Swarup et al., 1981; Leis & Kaplan, 1982; Li et al., 1984; Chernoff & Li, 1985). Several species of phosphotyrosyl-protein phosphatase with activity against phosphotyrosyl-casein, -IgG, -albumin and -p130^c-fos have been partially purified (Foulkes et al., 1983; Chernoff & Li, 1983; Shriner & Brautigan, 1984; Okada et al., 1986; Rotenberg & Brautigan, 1987). All have broadly similar properties to those found for the phosphatases active against autophosphorylated insulin and EGF receptors in the present work. Solubilization of the particulate fraction with 2% Triton X-100 quantitatively extracted the phosphotyrosyl-(insulin receptor) and -(EGF receptor) phosphatase activities (results not shown). ^32P-labelled EGF receptor was shown to be the preferred substrate, rather than ^32P-labelled insulin receptor, for cytosolic, particulate and calmodulin-dependent protein phosphatases. Selective dephosphorylation of tyrosine residues in the insulin receptor by phosphotyrosyl-protein phosphatase (e.g. as in Fig. 5) may facilitate elucidation of the function of insulin-stimulated serine phosphorylation of the insulin receptor.

The physiological role of calmodulin-dependent protein phosphatase as a phosphotyrosyl-receptor phosphatase was assessed. The assay of both native and calmodulin-dependent phosphotyrosyl-protein phosphatases in a single study against the same substrates permits the contribution of calmodulin-dependent protein phosphatase activity to total phosphotyrosyl-protein phosphatase activity to be calculated as follows. Tissue contents of calmodulin-dependent protein phosphatase are approx. 25, 12 and 5 mg/kg in both particulate and soluble phases of rat brain, liver and heart respectively (Wallace et al., 1980; Tallant & Cheung, 1983; Tallant et al., 1983; Ingebritsen et al., 1983). Efficient dephosphorylation of receptors required 320 μg of calmodulin-dependent protein phosphatase/ml activated by the physiological activators Ca^{2+} and calmodulin. Concentrations of particulate and soluble extracts that achieved the same rates of dephosphorylation contain at most 0.4, 1.2 and 3.6 μg of calmodulin-dependent protein phosphatase/ml for heart, liver and brain respectively. On this basis the contribution of calmodulin-dependent protein phosphatase to phosphotyrosyl-protein phosphatase activity is < 1.2%. Because of the possibility that the specific activity of the purified calmodulin-dependent protein phosphatase may differ from that in vivo [e.g. Chan et al. (1986) observed 4–5-fold variation in specific activity of the purified enzyme], it was important to confirm this small contribution independently. This was achieved by showing that Ni^{2+} inhibited the receptor phosphatase activities in cell fractions by 90–100%, whereas it activated calmodulin-dependent protein phosphatase, and by using Mn^{2+}/Ca^{2+}/calmodulin, which activated calmodulin-dependent protein phosphatase but failed to increase phosphotyrosyl-receptor phosphatase activity in cell fractions. Thus three lines of evidence have been obtained showing that in cell extracts phosphotyrosyl-protein phosphatases other than calmodulin-dependent protein phosphatase are the predominant (> 90%) phosphotyrosyl-(insulin receptor) and -(EGF receptor) phosphatases.

In conclusion, this work introduces Ni^{2+} as a new inhibitor of particulate and soluble phosphotyrosyl-(insulin receptor) and -(EGF receptor) phosphatases and illustrates its usefulness in demonstrating that the contribution of calmodulin-dependent protein phosphatase to the phosphatase activities is small. The finding that the vast bulk (75%) of native phosphotyrosyl-(insulin receptor) and -(EGF receptor) phosphatase activity is membrane-associated places the phosphatases in the correct proximity to attack membrane-bound receptors. Cytosolic phosphotyrosyl-protein phosphatases may act principally on soluble substrates of the membrane-associated tyrosine kinases, rather than the receptors themselves. Most phosphotyrosyl-protein phosphatases have so far been recovered from the cytosol (Foulkes et al., 1981, 1983; Chernoff & Li, 1983; Shriner & Brautigan, 1984), although Rotenberg & Brautigan (1987) have succeeded in partially purifying a catalytic fragment released from membranes by proteinases that has activity against a phosphotyrosyl residue in reduced and alkylated bovine serum albumin. The intact forms of phosphotyrosyl-protein phosphatases of membranes have not been isolated. Future work on the receptor phosphatases should place greater emphasis on characterizing the membrane-associated phosphotyrosyl-protein phosphatases.

Note added in proof (received 5 October 1988)

After submission of this paper, reports by Tonks et al. (1988a,b) appeared, describing the purification to apparent homogeneity of phosphotyrosyl-protein phosphatases from particulate and soluble phases of human placenta, with phosphotyrosyl-lysozyme as substrate. One of the phosphatases was shown to be able to dephosphorylate autophosphorylated insulin receptor, although the reaction was not characterized.

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